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A New Double Column HPLC Method for Rapid Separation of Fatty Acids

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Introduction

Knowledge of the content of the various fatty acids in blood plasma can be very helpful in the diagnosis and treatment of several diseases and disorders of metabolism. Moreover, the physiological importance of polyunsaturated fatty acids (PUFA) which have more than two double bonds per molecule have been reported recently. Thus, it is necessary to carry out fatty acid analysis in clinical fields.

Numerous methods are used for the determination of fatty acids. Gas liquid chromatography (GLC) of methyl esters of fatty acids is at present the most commonly employed. Thin layer chromatography is also a powerful tool but usually necessitates a second technique for quantitation. High-performance liquid chromatography (HPLC) seems ideally suited for the separation of long chain fatty acids with more than two double bonds[31]. However, complete separation of fatty acids by HPLC has not yet been obtained. Therefore, it was mandatory to devise a new precise HPLC method to determine fatty acids. This is the first paper to describe the successful separation of the biologically most important twelve C12–C22 fatty acids in a single-run analysis of 62 minutes.

Fatty acids of importance in human metabolism and nutrition are the monocarboxylic acids containing an even number of carbon atoms in a straight chain; those from 12 to 22 carbons. They are also characterized as saturated, monounsaturated or polyunsaturated acids. Naturally occurring fatty acids contain double bonds in the *sis* configuration. Thus, this paper describes a new method for rapid separation of 12 fatty acids with *cis* configuration and even carbon atoms.

Materials and methods

Apparatus

The chromatograms were obtained with a Shimadzu pump (LC-4A), equipped with fluorescence detector (RF-500LC, Shimdazu Co., Kyoto, Japan) at the wavelength of 365 nm

Key words: High-performance liquid chromatography (HPLC), Essential fatty acid (EFA) deficiency, Polyunsaturated fatty acids (PUFA), Total parenteral nutrition (TPN), Elemental diet (ED).
索引語：高速液体クロマトグラフィー，必須脂肪酸欠乏症，多価不飽和脂肪酸，完全静脈栄養法，エレメンタル・ダイエット.
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(excitation) and 412nm (emission), and fatty acid composition was calculated by determining the peak areas electronically by Chromatopac CR-2A (Shimadzu Co., Kyoto, Japan). The separation of fatty acids were carried out on two reversed phase C-8 columns, Zorbax C-8 (5 μm particle, 15 cm × 4.6 mm I.D., Shimadzu Co., Kyoto, Japan) and Lichrosorb RP-8 (10 μm particle, 25 cm × 4.0 mm I.D., Merck, West Germany) at a constant temperature of 35°C (CTO-2AS, Shimadzu Co.). The mobile phase was acetonitrile/water, and gradient elution technique was employed from 85/15 to 95/5, as depicted in Fig. 1.

Reagents

All chemicals used were of analytical reagent grade (Kanto Chemical Co., Tokyo, Japan) and were not further purified. Standard fatty acids were purchased from Nippon Chromato Kogyo Co. (Tokyo, Japan).

It was necessary to derivatize fatty acids because they have no characteristic fluorescence or strong UV absorption. Derivatization was carried out by incubating fatty acids with 9-anthryldiazomethane (ADAM) reagent[28] for one hour at ambient temperature and an aliquot was injected on HPLC.

Pretreatment of samples

Blood was withdrawn from antecubital veins after overnight fasting at 9:00 a.m. and centrifuged at 3000 rpm for 10 minutes. The serum was collected and stored at −20°C until required. All pretreatment procedures were performed in an air-conditioned laboratory room.

Extraction of total lipids

The procedure of Folch et al.[15] was mainly followed. Briefly, 0.1 ml of serum or bile was dripped into 3.9 ml of chloroform/methanol (2/1, V/V) in a glass-stoppered cylinder and stirred vigorously on a vortex-mixer for 1 minute. After standing for 30 minutes, 0.8 ml of saline was added and shaken vigorously on a vortex-mixer for 1 minute. After allowing to stand for 2 hours followed by centrifugation at 3000 rpm for 5 minutes, the upper methanol layer was removed and discarded and the lower chloroform layer was filtrated through 0.45 μm Millipore filter (Millipore Co., U.S.A.) after adding anhydrous Na_2SO_4 to remove water, and taken to dryness under a flow of nitrogen.

Tissue sample (0.1 g) was minced and added to 0.5 ml saline and homogenized using a Potter-Elvehjem homogenizer. Then, 4.5 ml chloroform/methanol (2/1, V/V) and 0.5 ml saline was added to the homogenates and homogenized using the homogenizer, and allowed to stand for 20 minutes. The upper methanol layer was removed and the lower chloroform layer containing total lipids were evaporated to dryness under a flow of nitrogen. The following procedures were the same as described for other biological samples.

Fractionation of total lipids

When total lipids were hydrolysed, this procedure was unnecessary. The total lipids were redissolved in 50 μl of chloroform and the half were spotted from a microsyringe on Silicagel-G

aluminum plate, Kieselgel 60 (20×20 cm, 0.2 mm thick, Merck, West Germany) and developed in the TLC glass chamber whose two sides were lined with Toyo filter paper (Tokyo, Japan). The developing solvents were petroleum ether/diethyl ether/acetic acid (80/20/1, V/V/V). After development the plate was dried and 2′,7′-dichlorofluorescein in ethanol (0.2%) was sprayed and visualized under ultraviolet light (360nm). Four distinct fractions, cholesterol ester, triglyceride, NEFA, phospholipid, in that order from the solvent front were identified, and R_f values were 0.85, 0.65, 0.37 and 0, respectively.

Hydrolysis of fractionated fatty acid esters

The mild-alkaline hydrolysis procedure by Dawson[11] was mainly followed. Each lipid fraction was removed with a knife and was put in round bottomed flasks with fitted glasss stoppers, and hydrolysed by 1 N-NaOH 0.25ml, methanol 4.5ml, and H_2O 0.25ml in a warmer bath maintained at 50°C for 30 minutes. Hydrolysis was stopped by addition of 0.3ml 1 N aqueous HCl, 2 ml H_2O, and 2 ml diethyl ether. Next 3 ml of petroleum ether was added into the flasks and vigorously shaken on a vortex mixer and allowed to stand until two complete layers were formed. This extraction of free fatty acids were repeated twice. Free fatty acid contained in the upper layer was taken and filtrated through 0.45 μm Millipore filter, and was taken to dryness in Pyrex conical vials under a flow of nitrogen in a warmer bath at 50°C.

Derivatization for HPLC

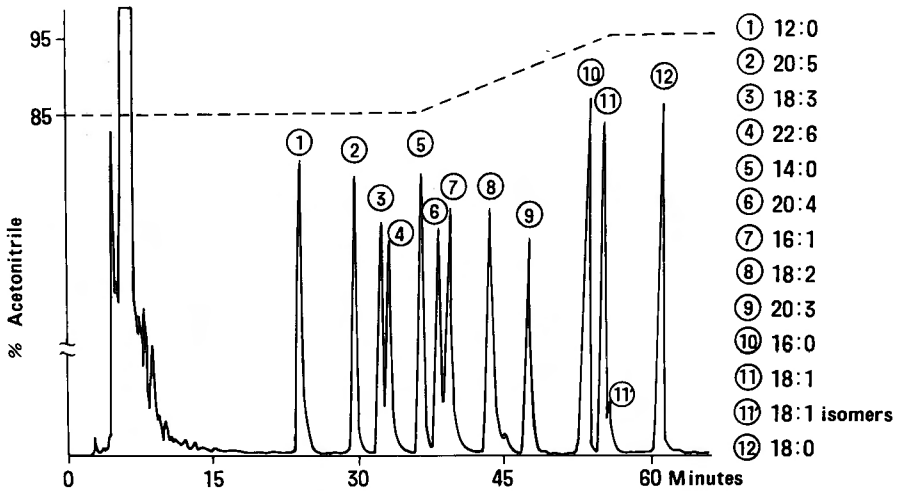
ADAM reagent was dissolved in ethanol (0.1%, W/V) immediately before use and stored in a refrigerator. Samples of free fatty acids were incubated with 50 μl of ADAM solution for 60 minutes at ambient temperature. ADAM derivatization was 80% in the initial 5 minutes and 100% within 1 hour at ambient temperature[28]. Five μl of the derivarized sample was injected on the HPLC system.

Results

1. Resolution of standard fatty acids

The order of elution and the resolution of a mixture of twelve ADAM derivatives of saturated, monoenoic and polyenoic fatty acids are given in Fig. 1 which was already presented in the short communication in 1982[32]. Other fatty acids, especially with short carbon chain occur in negligible amounts in human subjects. The blank was due to ADAM reagent and its break down products, but it did not disturb the separation or detection of the first eluting fatty acid, C12:0, and the base-line was stationary enough to permit detecting small quantity of all fatty acids. Separations between each fatty acid are satisfactory. Saturated fatty acids are separated according to chain length with the longer chains eluting last. The introduction of double bond decreased the retention time. As the number of double bonds in the hydrocarbon chain increased, the retention time decreased.

This was well illustrated with stearic C18:0 (peak 12), oleic C18:1 (peak 11), linoleic C18:2 (peak 8) and linolenic C18:3 (peak 3) acids all of which contain 18 carbon atoms with zero, one,



①Lauric, ②Eicosapentaenoic EPA, ③Linolenic, ④Docosahexaenoic, DHA
⑤Myristic, ⑥Arachidonic, ⑦Palmitoleic, ⑧Linoleic, ⑨Eicosatrienoic,
⑩Palmitic, ⑪Oleic, ⑫Stearic

Fig. 1. HPLC chromatogram of standard fatty acids.

two and three double bonds, respectively. The order of elution was dramatically opposite from that normally observed on polar GLC columns[20]. In contrast to GLC, in HPLC the longer the carbon chain and the higher the unsaturation, the earlier the fatty acid elutes. Early elution of biologically important PUFA, makes them quantitated accurately.

A single-run analysis required only 62 minutes. It was short enough to carry out this HPLC method practically.

2. Double-column method

On only one column it was impossible to obtain satisfactory separation among a critical pair which was composed of C14:0, C20:4 and C16:1. On a Zorbax C-8 column alone C14:0 and C20:4 were separated but C20:4 and C16:1 were not. On the contrary, on a Lichrosorb RP-8 column alone C20:4 and C16:1 wered separated but C14:0 and C20:4 were not, as shown in Fig. 2. Thus it was found that separation among these three fatty acids could be obtained by combining directly the two columns as shown in Fig. 1.

3. Sensitivity, calibration curve

Good linearity for each fatty acid was obtained in the range from 10 ng to 1000 ng (Fig. 3). The sensitivity was 10 to 100 times higher than the current GLC method following methylation or other HPLC methods with UV detection following phenacyl esterification. This high sensitivity permitted us to carry out fatty acid analysis at a very low concentration, in other words, requiring only a small volume of sample. It is possible to carry out the analysis with as little as 10 μl of serum. It is estimated that by increasing the detector sensitivity, 1 ng or less could be measured.

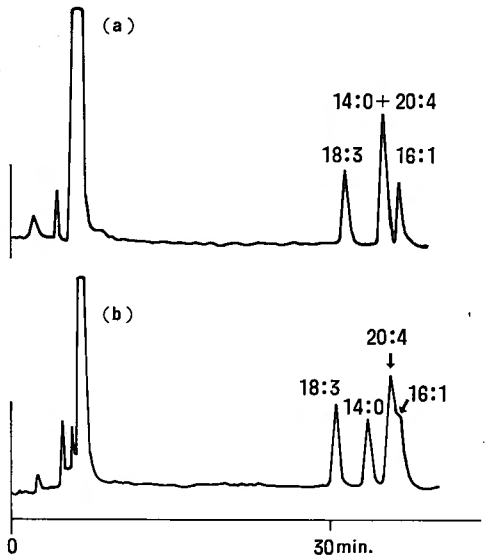


Fig. 2. Incomplete Separation among $C_{14:0}$, $C_{20:4}$, and $C_{16:1}$ on (a) Lichrosorb RP-8 (10 μm, 25 cm × 4.0 mml.D.) and (b) Zorbax OD-8 (5 μm, 15 cm × 4.6 mml.D.) Instrument: Shimadzu LC-4A, eluent: acetonitrile/water 85/15, flow rate: 1.0 ml/min.

4. *Reproducibility and accuracy*

The reproducibility of retention time and peak area for each fatty acid are shown in Table 1. The coefficients of variation of the retention time for within one day precision and day to day precision were in the range of 0.10 to 0.20%, and 0.24 to 0.52%, respectively. The coefficients of variation of the peak area for within one day precision and day to day precision were 6.00 to 10.6%, and 5.44 to 7.85%, respectively.

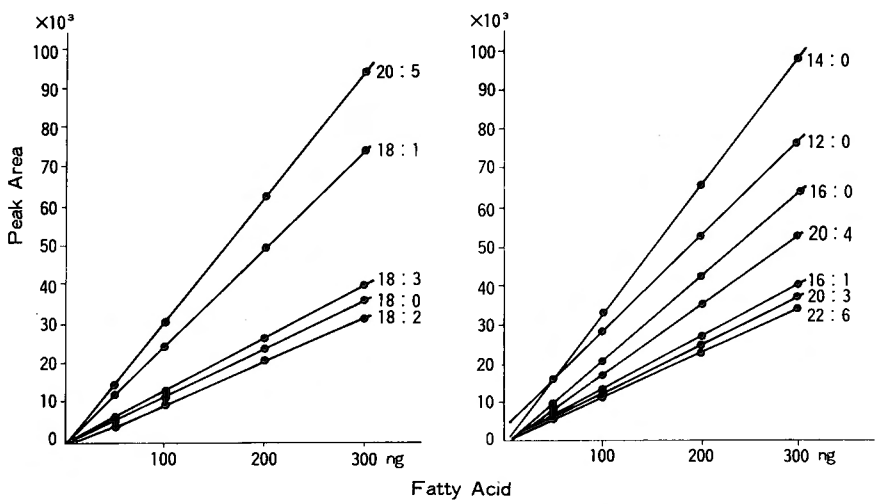


Fig. 3. Calibration curve for 12 fatty acids

Table 1. Reproducibility of retention time and peak area for each fatty acid.

	Retention time				Peak area	
	a		b		a	b
	R.T.(Minutes)	C.V. (%)	R.T. (Minutes)	C.V. (%)	C.V. (%)	C.V. (%)
① 12:0	26.34 ± 0.04	0.14	25.63 ± 0.11	0.43	6.25	6.87
② 20:5	31.69 ± 0.05	0.16	30.65 ± 0.16	0.52	7.02	7.07
③ 18:3	35.66 ± 0.06	0.18	34.46 ± 0.18	0.52	6.58	7.85
④ 22:6	37.14 ± 0.07	0.20	35.80 ± 0.21	0.58	6.86	6.40
⑤ 14:0	40.64 ± 0.07	0.17	39.25 ± 0.19	0.49	7.75	6.91
⑥ 20:4	42.97 ± 0.07	0.17	41.41 ± 0.25	0.59	9.50	7.08
⑦ 16:1	44.17 ± 0.08	0.18	42.67 ± 0.25	0.55	10.60	6.90
⑧ 18:2	47.97 ± 0.06	0.13	46.61 ± 0.21	0.45	6.61	6.98
⑨ 20:3	51.78 ± 0.07	0.13	50.55 ± 0.18	0.36	7.72	5.44
⑩ 16:0	56.03 ± 0.06	0.10	54.99 ± 0.15	0.26	9.89	7.42
⑪ 18:1	57.95 ± 0.06	0.10	56.97 ± 0.14	0.24	7.34	5.63
⑫ 18:0	65.32 ± 0.08	0.12	64.46 ± 0.18	0.27	6.00	7.06

(mean ± S.D.)

a : within a day precision, b : day to day precision

5. Effect of temperature

Retention times and separations were markedly affected by column temperature. The higher the column temperature, the earlier every fatty acid eluted, the poorer the resolution between C18:3 and C22:6 and among C14:0, C20:4, and C16:1 became. Resolution factors among these fatty acids at various column temperatures were compared and optimum tempeature was found to be 35°C.

Discussion

Treatment and prevention of malnutrition has been major problems in the surgical field. Since the introduction of total parenteral nutrition (TPN) by Dudrick in the late 1960's and elemental diets (ED) in the early 1970's, many patients suffering malnutritional states such as intestinal fistula, inflammatory bowel disease, and chronic pancreatitis have been managed sufficiently by TPN or ED, being supplied with enough calories without oral intake. Hyperalimentation not only improves the nutrituonal state but also promotes wound healing and closure of fistula, and suppresses inflammation such as Crohn's disease and pancreatitis, but has brought a serious complication, namely, essential fatty acid (EFA) deficiency[37], and this deficiency can be prevented and treated by intravenous administration of fat emulsion. Therefore, fat emulsion is generally administered to all patients, except contraindicated patients to supply linoleic acid which is the major essential fatty acid in preventing its deficiency. If they are

maintained exclusively by parenteral or enteral nutrition, the minimum requirement of linoleic acid is presumed to be 4% of the total calories[21] but this is still under debate[9]. However, babies and infants require more linoleic acid than adults for normal body growth and mental development. At present, 200 ml of fat emulsion per day or 500 ml of it every other day is administered empirically[38]. This dose supplies linoleic acid approximately as 4% of the total calories, 2000 Cal per day being supplied. However, if a much larger doses of EFA than required is administered for a longer period, some overloading symptoms might appear.

Although classical symptoms of EFA deficiency which was first described by Burr and Burr are not commonly encountered, biochemical EFA deficiency is commonly seen in blood fatty acid patterns of nutritionally depleted patients. Some fatty acids have been put in and out EFA, but linoleic acid and arachidonic acid have been constantly considered to be EFA. Diagnosis of EFA deficiency depends on fatty acid analysis of blood, and its current cditeria are as follows:

1. decrease in linoleic acid and arachidonic acid
2. appearance of eicosatrienoic acid C20:3
3. triene/tetraene ratio (20 3/20 : 4) above 0.4
4. increase in monoenes: C16:1, C18:1.

EFA deficiency can be improved or prevented by the administration of fat emulsions containing about 50% of calorie as linoleic acid. However, the dose of fat emulsion is not determined for the individula case. Thus, it is mandatory to measure the serum concentration of each fatty acid as well as triene/tetraene ratio, and determine the ideal dose of fat emulsion.

For these reasons it is necessary to carry out fatty acid analysis but, in fact, this is not always done. This tendency was partly due to deficiencies of the employed analytical tools per se, GLC or TLC.

Applications of HPLC have been increased greatly during the past decade. The increase in utilization is not very surprising in view of the vast power offered by modern HPLC for resolving various types of compounds of biochemical interest.

The efforts of pioneers who hve analysed fatty acids by HPLC have been directed at improving resolution of components and the sensitivity of detection. Attempts to improve sensitivity have included a variety of derivatives and these are covered by Cooper and Anders[8]. Other HPLC methods were limited because of poor sensitivities and tedious procedures for derivatization in addition to incomplete separation[10,18,27,29]. Refractive index detectors are relatively insensitive to fatty acid concentrations at the nanogram level[34]. Ultraviolet (UV) detectors can be sensitive to such low concentrations, but most fatty acids do not absorb UV radiation at the wavelength of 254 nm employed in many instruments. Consequently, conversion of fatty acids to UV-sensitive derivatives is employed. Suitable derivatives include the corresponding benzyl, p-bromophenacyl[12,14], p-nitrobenzyl[7], p-nitrophenacyl, and phenacyl esters[17]. But reaction conditions required are complicated and time-consuming. Moreover, Wood reported that phenacyl derivatizatives of monoenoic acids were also shown to undergo *cis-trans* isomerization when exposed to ultraviolet light[41]. Moreover, samples that contained a large excess of reagent deteriorated noticeably within one week and were more than 75%

destroyed within one month, even when stored at −20°C[41].

Satisfactory separation had been reported to be difficult and incomplete or impossible by other HPLC method in much longer time, 3 hours or more[3,25,36], even with gradient elution technique. The most difficult separations were between C18:3 and C22:6, and among C14:0, C20:4, and C16:1, and no satisfactory separations for these fatty acids have been reported, hitherto. Recently, Halgunset[19] and Wood[41] reported HPLC methods for resolutions on reversed μ-Bondapack C-18 columns with the analysis time required within 60 minutes. However, neither of them referred to EPA C20:5 and DHA C22:6 which are considered to play the most important biological roles. Moreover, the resolution between C14:0 and C16:1 was incomplete in the former, and no peak of C16:1 was identified in the latter.

There have been several studies on the HPLC method which employed two (60 cm)[3,25] or three (90 cm)[1] columns for improved separation of fatty acids. But all of them connected the same columns whose packed particles and inside diameter were same. Moreover, they required long analysis time (two to four hours), and separations between critical pairs were incomplete. Thus, this is the first paper describing the connection of two columns which differ in packed particles, inside diameter and length, and thus obtained improved separation.

Unsaturated fatty acids comprise geometrical and positional isomers and their separation is also possible by the author's HPLC[33]. Distinguishing among geometrical (*cis* and *trans*) and positional isomers of the olefinic acid by HPLC was reported[23,35], but not by routine GLC. More sophisticated GLC employing glass capillary column, can separate isomers[22,26,35,39]. However, it is expensive and requires special technical training, so its clinical applicability is limited. The resolution of C18 : 1-isomers are given as a peak 11′ in Fig. 1. This isomer was obtained from the phospholipid fraction of Venolipid which was a newly developed fat emulsion. The resolution of C18 : 1 geometrical isomers by reverse-phase HPLC is not unique to the author. Some excellent separations of phenacyl derivatives of *cis* and *trans* monoene isomers have been demonstrated by Borch[1] and by Bussel[4] on very long columns (60–90 cm) that required up to 4 hours for analysis. Recently, equal resolution with one 25 cm column in a paractical time (52 minutes) was reported by Wood[41]. Svenson et al.[35] also recently reported retention times for a series of *cis* and *trans* C18 : 1-isomers separated on a single column as methyl esters. Analysis time required is several minutes shorter, but this positive aspect of the methyl esters is offset by the difficulty in trying to obtain quantitative data on mixtures of esters using either the UV detector in the region of 195 to 206 nm or the refractive index detector[35].

Short and medium chain fatty acids (C4:0 to C12:0) occur in relatively small amounts and do not play important physiological roles. On the contrary, long chain fatty acids, especially PUFA with more than two double bonds are of interest due to their physiological significance. In GLC the longer the carbon chain and the higher the unsaturation, the later the fatty acid elutes. Thus, PUFA such as eicosapentaenoic acid (EPA) C20:5 and docosahexaenoic acid (DHA) C22:6 can not be detected due to technical problems if they are present in small amounts.

Recently, attention has been paid to EPA by many workers because it is expected to reduce platelet aggregation and thus prevent thrombose formation and ischemic heart disease. The

mechanism of reduced platelet aggregation is still unclear but it appears that EPA released from phosphatidylcholine in platelet is converted to prostaglandin and thromboxane which inhibit platelet aggregation as a net result. Epidemiological data[13] of Eskimo in Greenland showed that their bleeding time was prolonged and ischemic heart disease was rare compared to the Canadian and American populations. Fatty acid analysis revealed that EPA was dominant in Eskimo but in the American and Canadians arachidonic acid was dominant. From this view point it is important to measure EPA content. The physiological importance of DHA remains to be clarified. This is due to the absence of analytical tools which are clinically applicable. As already mentioned, GLC is not a suitable tool for the analysis of PUFA, especially DHA which eluted last among the physiologically important fatty acids in human subjects because of its long carbon chain and high unsaturation.

The use of this HPLC method may contribute to the elucidation of physiological roles of DHA. From the proposed physiological importance of PUFA such as EPA and DHA, application of HPLC for fatty acid analysis would surely be increased.

It has been noted by workers employing GLC that eicosatrienoic acid C20:3 can not be detected normally and its presence suspected EFA deficiency[5,6,30,40]. However, by HPLC, C20:3 could be always detected in healthy volunteers at an average level of 2.0% or 2.7 mg/dl in the serum phospholipid fraction as shown in Fig. 4. The failure of detection in GLC was partly due to the method of fatty acid analysis of total lipids. Because other fractions contain C20:3 at the level of less than 0.1%. This obscured C20:3 in phospholipid fraction by dilution effect. Another reason is the limit of detection. As mentioned above, GLC is less sensitive than the fluorescence detection employed in the author's HPLC method.

To prevent oxidation, butylhydroxytoluene (BHT) is usually added as an antioxidant in all organic solvents at the concentration of 0.1% W/V. In GLC, BHT is detected at nearly the same retention time of methyl-C16:1, one of the important fatty acids which increase in EFA deficiency. Thus, GLC can not be carried out in the presence of BHT due to its interference with C16:1 determination. This is one of the reasons why comparative quantitative study between HPLC and GLC was not carried out. In performing fatty acid analysis it is also necessary to do all procedures under as low a temperature as possible and in the absence of oxygen to prevent autooxidation of PUFA. In GLC conditions, oven temperature gradient up to 230°C is employed to shorten analysis time. Such a high temperature might induce hydrogenation of double bonds and migration of double bonds along the carbon chain. In HPLC conditions described here, the column temperature was maintained at 35°C and all reactions were carried out below 50°C.

This rapid and precise HPLC method will be applicable to diverse fields, including medical and agricultural fields.

1. HPLC will be applicable to blood to assess EFA status and diagnose and treat various disorders, for example, EFA deficiency and metabolic disorders of lipids.

2. Application to analysis of bile: The formation of cholesterol gallstones is thought to result partly from changes in the relative composition of bile lipids. Changes in fatty acid composition in biliary phosphatidylcholine will influence the detergent effect of phosphatidylcholine in bile,

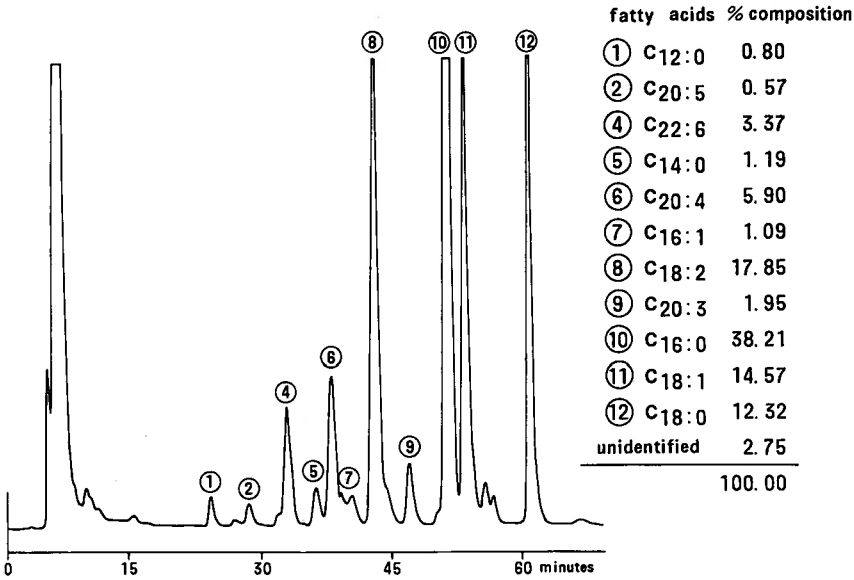


Fig. 4. HPLC chromatogram of serum phospholipid in a 46 years old male, Triene/Tetraene ratio: 0.33.

and on cholesterol gallstone formation. HPLC will also be applicable to bile to determine fatty acid composition of cholesterol gallstone.

3. Fatty acid composition of any tissue lipids can be determined.

4. Application to food stuff: A strong correlation between fatty acid composition and dietary fat and ischemic heart disease has been established. HPLC will also be a valuable tool for eluci-

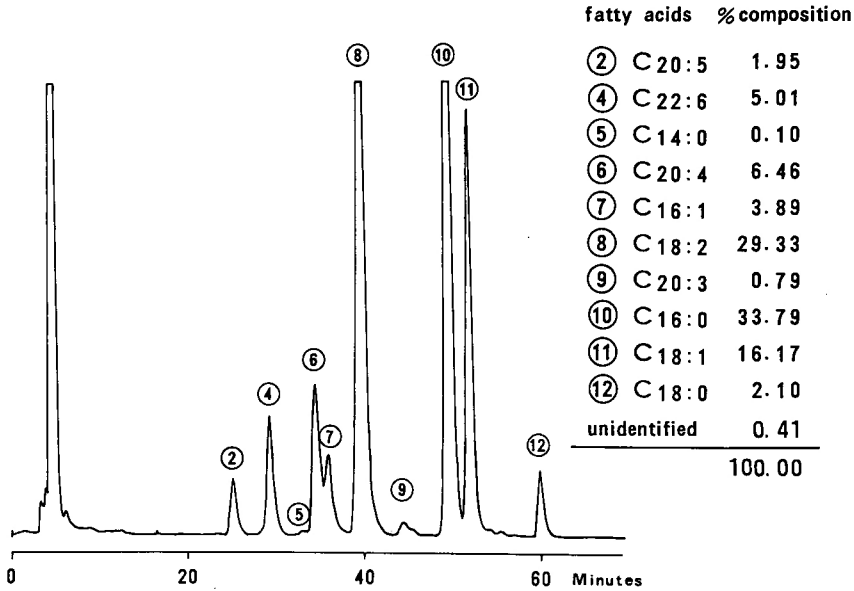


Fig. 5. Fatty acid composition in hepatie bile.

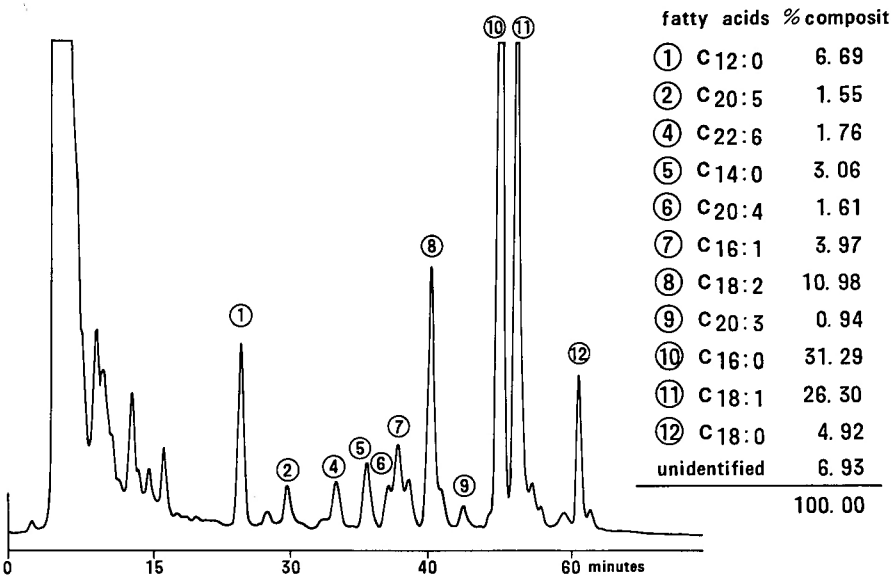


Fig. 6. HPLC chromatogram of adipose tissue in a 60 years old female.

dating the interrelationship between dietary fatty acid composition and various disorders and the development of the preventive medicine. Each representative HPLC chromatogram of the biological applications is given in Fig.s 4 to 7. Satisfactory base-line separations are well maintained as shown in the chromatogram of standard mixtures.

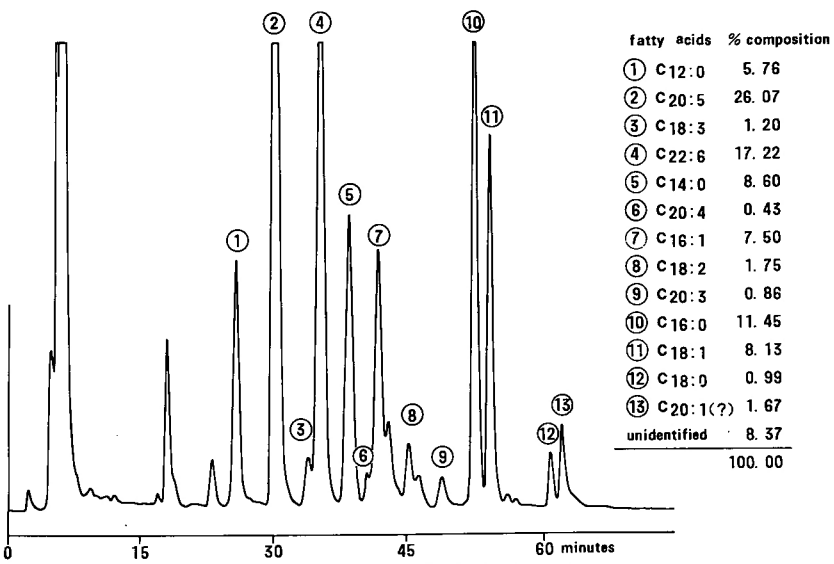


Fig. 7. HPLC chromatogram of SAN OMEGA.
SAN OMEGA is a purified product from marine oils.

Conclusion

1. The newly devised HPLC method described here has several advantages compared with previously published methods, including GLC and HPLC.

2. The chromatographic procedure is simple and fast (62 minutes), and the retention times are very reproducible. It is thus a simple, sensitive and reliable method for the separation of biologically relevant twelve C12–C22 fatty acids.

3. Complete separations between critical pairs, C18:3 and C22:6, and C14:0, C20:4 and C16:1 were obtained. These separations have not been reported in such a short analysis time by others.

4. Its clinical application to blood, bile, tissue and food stuff would surely be increased.

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和文抄録

新 HPLC 法による迅速脂肪酸測定法

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外科領域においては1960～70年代の TPN, ED の導入により栄養管理法に飛躍的進歩がもたらされた反面，臨床的にも必須脂肪酸欠乏症という重篤な合併症が出現するに至り，それら脂質代謝異常の診断と治療に血中脂肪酸の測定は必須である．しかも，臨床症状を呈さない生化学的必須脂肪酸欠乏症は，無脂肪栄養管理下では早くも開始4日目から指摘されており，臨床症状の発現以前に血中脂肪酸の測定によって早期診断，症状発現防止を行なうのが望ましい．しかるに，現在繁用されている GLC 法は臨床応用するには，感度・定量性の面で問題があり，又操作の煩雑さもあり，広く応用されていない．

著者は，臨床の実際に応用しうる迅速・正確でかつ再現性の高い HPLC 法を以下の如く，確立した．その結果，

著者の開発した HPLC 法が従来の GLC 法に優る点として，

1)　近年動脈硬化症や血栓症予防などの面より注目を集めている EPA $C_{20:5}$ をはじめとして DHA $C_{22:6}$, 必須脂肪酸欠乏症の診断に欠かせない $C_{20:3}$, $C_{20:4}$ などの多価不飽和脂肪酸が迅速，正確に測定しうること．すなわち，この HPLC 法では二重結合が増加する程，GLC 法とは逆に，早期に溶出するのが利点である．

2)　測定感度を極めて高めることができ，ng レベルでも測定可能となったこと．すなわち，10 μl の血清や胆汁試料でも分析できるようになった．

3)　特殊な GLC 法でない限り，分離不能であった異性体の分離も可能となったこと．すなわち，二重結合を含む脂肪酸には立体 (cis, trans) ならびに位置異性体が存在し，最近，特に天然に存在する cis 体以外の異性体の生体内代謝が注目されるようになったが，この異性体の分離も HPLC 法の条件変更により容易となった．

4)　さらに，キャピラリーカラムを使用すると異性体の分離は GLC 法でも確かに可能であるが，操作が煩雑な昇温グラジエントを用い，200°C 以上の高温にする必要がある．この操作により，多価不飽和脂肪酸の二重結合の位置を変化させたり，酸化を導入したりする可能性がある．しかし，著者の HPLC 法ではすべて 50°C 以下で操作するため，この心配が全くないこと．

5)　分析に要する時間も62分と短く，一回の注入で，生体内に存在する主な脂肪酸12種がすべて測定できるようになったこと，などが挙げられる．

さらに，既報の HPLC 法では，単に同一のカラムを3本接続し，分析時間 3～4 時間を費やしても $C_{18:3}$-$C_{22:6}$ および $C_{14:0}$-$C_{20:4}$-$C_{16:1}$ 間の分離は不可能であったが，Zorbax OD-8 (5 μm), Lichrosorb RP-8 (10 μm) という充填剤粒子径が異なり，かつ長さおよび径も異なる2本のカラムを直列に接続するという著者独自の発案により，これら2群の脂肪酸相互間の満足すべき分離条件を開発した．

以上，著者の開発した HPLC 法は，迅速・正確で，再現性が高く，特に多価不飽和脂肪酸の分析に優れている点より，

①　必須脂肪酸欠乏症の予防と早期診断に $C_{18:2}$, $C_{20:3}$, $C_{20:4}$ を定量し，$C_{20:3}/C_{20:4}$ 比を求めたり，②　食品や血清はもちろんのこと，胆汁や組織内脂質における EPA, DHA の測定も正確・迅速にでき，今日まで未解決なこの種の脂肪酸の詳細な体内動態を明白にし得るなど，今後，就中，臨床分析に有用と考える．